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(54) Process for the preparation of a unit for the determination of residues of antibiotics and sulphas in biological liquids, test tube or set of test tubes thus prepared and process for carrying out the determination.

(57) Process for the preparation of a unit for the determination of residues of antibiotics and sulphas in biological liquids, the unit thus prepared and process for carrying out the determination. Spores of a microorganism showing a broad sensitivity for the antibiotics and sulphas to be tested are introduced into an agar solution so that they cannot germinate but stay alive, while by addition of a small amount of trimethoprim the sensitivity for sulphas is increased, whereafter the agar solution is allowed to solidify in tubes of preferably transparent material. The unit is very useful for the determination of residues of antibiotics and sulphas in, e.g. milk by less skilled persons.

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TITLE MODIFIED
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Analysis unit

The invention relates to a process for the preparation of a unit for determining residues of antibiotics and sulphas in biological liquids, such as milk, meat juice, serum and urine. The invention further relates to the unit itself, prepared by means of the process according to the invention, and to the large scale application of the process.

A similar process has been described by Gudding, Acta Vet. Scand. 17 (1976) pages 458 to 464, using plates with an agar medium in the manner as described by Galesloot et al, Netherlands Milk and Dairy Journal 16 (1962) pages 89 to 95, in which, however, the agar medium has been adapted to enable determinations of sulphas by the addition of trimethoprim (Merck Index 9th Ed. No. 9377). Sulphas are, generally speaking, compounds with a substituted or unsubstituted SO_2NH_2-group at the para site of a substituted or unsubstituted aniline nucleus, such as sulphamethoxazole [4-amino-N-(5-methyl-3-isoxazolyl)benzenesulphonamide]. In this process use is made of, inter alia, the thermophilic microorganism Bacillus stearothermophilus var. calidolactis, which is preferably incubated at about 60°C in order to avoid interferences by microorganisms present in the sample to be tested. In addition to the fact that fresh plates have to be prepared for each determination, the result of the test can be read not earlier than 6 hours after starting it.

The practice, however, needs a quicker test, using ready-for-use requirements, and giving a result within a few hours. In addition, the practice needs a test, which does not necessarily involve qualified laboratory personnel, and which may be carried out by, e.g. the truck driver transporting the milk from the farmer to the factory.

British Patent Specification No. 1,467,439 describes a test starting indeed from ready-for-use requirements. That test gives a result within $1\frac{1}{2}$ to 4 hours, generally within 2 to 3 hours,

and may be carried out by unqualified personnel, but the test is suitable for the determination of only antibiotics in biological liquids, such as milk, meat juice, serum and urine, and shows too little sensitivity for the determination of sulphas.

According to the invention the test described in British Patent Specification 1,467,439 may be adapted for the determination of sulphas, and with the same test-duration of 2 to 3 hours.

Therefore the invention provides a process for the preparation of a unit for the determination of residues of antibiotics and sulphas in biological liquids, such as milk, meat juice, serum or urine, characterized in that spores of a microorganism, showing a broad sensitivity for the antibiotics and sulphas to be determined, are introduced into an optionally buffered agar solution in such a manner, that they cannot germinate by lack of nutrients and/or by low temperature, but stay alive, and in such an amount, that the microorganism, in the presence of nutrients and by incubation at a temperature at or near the optimal temperature for the growth of the microorganism, will grow in a short time to such an extent, that growth is observable by means of an indicator, while further, by addition of an amount of trimethoprim [2,4-diamino-5-(3,4,5-trimethoxybenzyl)-1,3-pyrimidine] the sensitivity for sulphas is increased but the speed is not or is substantially not influenced, the agar solution is then allowed to solidify in tubes and, if the nutrients for the growth of the microorganism are prepared separately, the indicator is added either to the agar solution or to the nutrients.

The test tubes prepared according to the invention enable to obtain a result, within $1\frac{1}{2}$ to 4 hours, generally within 2 to 3 hours, whether a sample of biological liquid contains or does not contain an antibiotic or a sulpha in excess of a predetermined concentration. It has surprisingly been found that the test succeeds also without the adaptation of the medium, as reported by Gudding, so that, for instance, the medium described in British Patent Specification No. 1,467,439 may be used as such.

It has also surprisingly been found that the time for reading the result is not necessarily extended. Thus, by choosing a suitable trimethoprim concentration and reading on acid formation or reduction, for instance according to the vertical diffusion test method of British Patent Specification No. 1,467,439, the test time according to that method may be maintained. It is appreciated that Gudding used a trimethoprim concentration of 0.25 µg/ml, whereas, according to the invention, the trimethoprim concentration may be lower, as indicated hereinafter.

Trimethoprim appeared not or substantially not to influence the keepability of the spores. Furthermore, such a unit may be storable for more than a year.

Examples of tubes useful for the purpose of the invention are transparent tubes, single or in a set or combined to a block of translucent material provided with a number of holes shaped therein.

The nutrients necessary for the growth of the microorganism are preferably included in a tablet or in a disc of filter paper or anything like that. The tablet or the filter paper disc is placed on the agar medium before carrying out a test. Nutrients, e.g. in a tablet may also be included in the test tube beforehand, whereby preferably measures are taken to avoid moisture transport from the agar medium into the tablet. This may be done, e.g. by coating the tablet with a moisture-resistant layer, for example a wax, which coating must disappear during the test. A wax having a melting temperature of 35 to 55°C, preferably 40 to 45°C, is suitable for that purpose. The nutrients must contain at least an assimilable carbon source, e.g. glucose, an assimilable nitrogen source, e.g. peptone, and a source of growth factor and minerals, e.g. yeast extract. If the nutrients are included in the agar medium the unit should be stored at temperatures below those where the spores germinate (2 to 10°C).

The indicator used is an acid-base indicator for a pH of about 5.5, preferably bromocresolpurple or phenolred, or a redox indicator, preferably 2,3,5-triphenyltetrazolium chloride or Brilliant black.

Spores of, preferably, thermophilic spore-forming bacteria which are sufficiently sensitive for the compounds to be tested, may be used for the invention. A suitable spectrum of sensitivities for the various antibiotics and sulphas may be obtained by using a single bacterium strain or a mixture of bacteria. Spores useful for the invention are those from spore-forming bacilli showing a sufficient sensitivity for the antibiotics and sulphas to be tested, such as Bacillus calidolactis (Hussong et al, J. Bact. 15 (1928) pages 179 to 188), Bacillus subtilis, Bacillus megaterium, Bacillus cereus, Bacillus stearothermophilus (Bergey's Manual of Determinative Bacteriology, 7th Ed. (1957) pages 613 to 693), the thermophilic bacilli described by Galesloot et al, Netherlands Milk and Dairy Journal 13 (1959) pages 155 to 179, Bacillus stearothermophilus var. calidolactis, described by Mol, Netherlands Milk and Dairy Journal 23 (1969) pages 153 to 162 and Bacillus calidolactis Strain C 953 from the Netherlands Institute for Dairy Investigation (Nederlands Instituut voor Zuivel-Onderzoek) at Ede (Galesloot et al, Netherlands Milk and Dairy Journal 16 (1962) pages 89 to 95). The spores of the strain Bacillus stearothermophilus var. calidolactis, deposited at the Laboratory of Microbiology of the Technical University of Delft under number LMD 74.1 - where the strain is available to the public - is preferably used. Another successful strain is the strain Bacillus stearothermophilus ATCC 7954. These microorganisms are very sensitive for penicillins. They are growing fast and show the additional advantage of their optimal growing temperature being so high that other microorganisms generally do not grow, resulting in only a small chance of those microorganisms being interfering. Preferably, the microorganism further shows a high sensitivity for other antibiotics.

The medium according to the invention contains, for instance, about 10^5 to about 10^8, preferably 10^6 to 10^7, spores of the microorganism per ml of the agar medium. The preparation of the spore-containing agar medium is further described in British Patent Specification No. 1,467,439.

The amount of trimethoprim, used according to the invention, is suitable for the determination of 0.05 to 2 µg/ml of most of the generally used sulphas in the sample. Suitably about 10 to about 120 µg, preferably 20 to 60 µg, of trimethoprim are used per litre of the medium.

The invention further relates to a test tube or a set of test tubes, which may be combined to a block of test tubes, for the determination of residues of antibiotics and sulphas in biological liquids, such as milk, meat juice, serum and urine, obtained by means of the process according to the invention. For the design of a set of test tubes, cf. British Patent Specification No. 1,467,439.

Another feature of the invention is a process for the determination of residues of antibiotics and sulphas in biological liquids, such as milk, meat juice, serum and urine, characterized in that a predetermined amount of the sample to be tested is placed in the tube obtained according to the invention, and is left there or removed after a sufficiently long time, e.g. 15 to 30 minutes for the diffusion of the residues of antibiotics and sulphas, subsequently if necessary the nutrients are placed on the agar medium, and the contents of the test tube are incubated at or near the optimal temperature for the microorganism during a predetermined period after which the indicator-colour is observed, indicating the presence or absence of an antibiotic and/or sulpha above a certain minimum concentration. The test is very simple to be carried out, so that qualified personnel is not necessary for the test. The determination may be done in $1\frac{1}{2}$ to 4 hours, preferably 2 to 3 hours, after starting the test, which is markedly shorter than for the method described by Gudding.

Claims.

1. Process for the preparation of a unit for the determination of residues of antibiotics and sulphas in biological liquids, such as milk, meat juice, serum or urine, characterized in that spores of a microorganism, showing a broad sensitivity for the antibiotics and sulphas to be determined , are introduced into an optionally buffered agar solution in such a manner that they cannot germinate by lack of nutrients and/or by low temperature, but stay alive, and in such an amount that the microorganism, in the presence of nutrients and by incubation at a temperature at or near the optimal temperature for the growth of the microorganism, will grow in a short time to such an extent that growth is observable by means of an indicator, while further, by addition of an amount of trimethoprim [2,4-diamino-5-(3,4,5-trimethoxybenzyl)-1,3-pyrimidine] the sensitivity for sulphas is increased but the speed is not or is substantially not influenced, the agar solution is then allowed to solidify in tubes and, if the nutrients for the growth of the microorganism are prepared separately, the indicator is added either to the agar solution or to the nutrients.

2. Process according to claim 1, characterized in that the nutrients are included in a tablet or filter paper disc to be placed on the agar before carrying out the test.

3. Process according to claim 1 or 2, characterized in that the indicator is an acid-base indicator, preferably bromocresol-purple or phenolred, or a redox indicator, preferably 2,3,5-triphenyltetrazolium chloride or Brilliant black.

4. Process according to any one of the preceding claims, characterized in that the spores of the microorganism are derived from Bacillus stearothermophilus, Bacillus subtilis, Bacillus megaterium or Bacillus cereus, preferably Bacillus stearothermophilus var. calidolactis (LMD 74.1).

5. Process according to claim 4, characterized in that about 10^5 to about 10^8, preferably 10^6 to 10^7 spores per ml of agar medium are used.

6. Process according to any one of the preceding claims, characterized in that about 10 to about 120 µg, preferably 20 to 60 µg, of trimethoprim per ml of medium are used.

7. Process according to any one of the preceding claims, characterized in that transparent test tubes, single or in a set or combined to a block of translucent material provided with a number of holes shaped therein, are used.

8. Test tube or set of test tubes, which may be combined to a block of test tubes, for the determination of residues of antibiotics and sulphas in biological liquids, such as milk, meat juice, serum or urine, obtained by means of the process according to any one of the preceding claims.

9. Process for the determination of residues of antibiotics and sulphas in biological liquids, such as milk, meat juice, serum and urine, characterized in that a predetermined amount of the sample to be tested is placed in a tube obtained according to the invention, and is left there or removed after a sufficiently long time, e.g. 15 to 30 minutes for the diffusion of the residues of antibiotics and sulphas, subsequently if necessary the nutrients are placed on the agar medium and the contents of the test tube are incubated at or near the optimal temperature for the microorganism during a predetermined period after which the indicator-colour is observed, indicating the presence or absence of antibiotic and/or a sulpha above a certain minimum concentration.

10. Process according to claim 9, characterized in that the incubation time is between $1\frac{1}{2}$ to 4, preferably 2 to 3 hours.

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EUROPEAN SEARCH REPORT

Application number

EP 79 20 0277

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim
	CHEMICAL ABSTRACTS, Vol. 83, no. 21, 24 November 1975, abstract: 176783k, page 400 COLUMBUS, OHIO, USA & NETH. MILK DAIRY JOURNAL, 1975, 29, no. 1, pages 16-34 J.L. VAN OS et al.: "Diffusion test for the determination of antibiotic residues in milk" * page 400, first column, complete abstract *	1,3,4

	CHEMICAL ABSTRACTS, Vol. 79, no. 17, 29 October 1973 abstract: 103722x, page 311 COLUMBUS, OHIO, USA & NORD. VETERINAERMED. 1973, 25, (4-5), 248-55 R. GUDDING et al.: "Detection of sulfonamides in milk" * page 311, columns 1-2, complete abstract *	1,4,6

D	GB - A - 1 467 439 (GIST-BROCADES) * complete document *	1-10

A	US - A - 3 126 325 (G.M. POOLE) * columns 2,3 *	1,4

	JOURNAL OF DAIRY SCIENCE, Vol. 50, no. 9, 1967 MAMPAIGN, ILLINOIS, USA J.M. PALMER et al.: "Simple ultra- ./.	1

CLASSIFICATION OF THE APPLICATION (Int. Cl.³)

G 01 N 33/16

TECHNICAL FIELDS SEARCHED (Int.Cl.³)

G 01 N 33/16
33/02
33/04
C 12 K 1/00

CATEGORY OF CITED DOCUMENTS

X: particularly relevant
A: technological background
O: non-written disclosure
P: intermediate document
T: theory or principle underlying the invention
E: conflicting application
D: document cited in the application
L: citation for other reasons

&: member of the same patent family, corresponding document

☒ The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
The Hague	22-08-1979	GANEFF

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DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int. Cl.³)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
	sensitive test for detecting penicillin in milk" * pages 1390-1394 * ---------		TECHNICAL FIELDS SEARCHED (Int. Cl.³)